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(19) (CA) **CANADIAN PATENT** (12)

(54) PROCESS FOR PREPARING HUMAN PLASMA FRACTIONS CONTAINING IMMUNE GLOBULIN (IGG)

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TITLE: AN IMPROVED PROCESS FOR PREPARING HUMAN PLASMA FRACTIONS CONTAINING IMMUNE GLOBULIN (IgG)

ABSTRACT

An improved process for preparing human plasma fractions containing immune globulin (IgG) which is suitable for intravenous use comprises the use of a dilute solution containing IgG as starting material, preferably obtained as an eluate from the chromatographic separation of plasma on a DEAE-Sephadex or a QAE-Sephadex column. The dilute solution containing IgG is treated with a mixture of sodium chloride and glycine and the dilute solution thus obtained is subjected to ultrafiltration to provide a concentrated solution containing IgG. The latter solution may, if desired, be freeze-dried to provide a solid composition of matter containing IgG.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:-

1. A process for the manufacture of an immune globulin (IgG) preparation which is suitable for intravenous use which comprises treating a dilute solution containing IgG with a mixture of sodium chloride and glycine and subjecting said dilute solution to concentration by means of ultrafiltration to provide a concentrated solution containing IgG.

2. The process of claim 1 wherein the dilute solution containing IgG used as starting material is an eluate obtained by a chromatographic procedure from a column containing an ion-exchange resin.

3. The process of claim 2 wherein the ion-exchange resin is DEAE-Sephadex A-50 or QAE-Sephadex A-50.

4. The process of claim 3 wherein human plasma which may or may not be prior diluted with water is subjected to chromatographic separation on the ion exchange column and the column is then eluted with an equilibrating buffer of 0.025 M phosphate buffer at a pH of 7.5.

5. The process of claim 1 wherein the dilute solution of IgG contains a high-titer of antibodies selected from the group consisting of Rh antibodies, rabies antibodies, tetanus antibodies and Zoster antibodies.

6. The process of claim 1 wherein the dilute solution of IgG is prepared from normal human plasma to provide standard or normal human immune serum globulin (HSIG or NSIG).

7. The process of claim 1 wherein the mixture of sodium chloride and glycine added to the dilute solution of IgG is such that the concentration of sodium chloride in said di-

lute solution is 0.15 M and the concentration of glycine in said dilute solution is 0.1 M prior to ultrafiltration.

8. The process of claim 1 wherein the ultrafiltration is carried out by means of a Millipore Pelicon System using a PTGC 000 05 cassette membrane (NMWL of 10,000/5 ft^2).

9. The process of claim 1 wherein the ultrafiltration concentrates the dilute solution containing IgG to a volume which is from about 1/10 to about 1/100 of the volume of said dilute solution before said concentration.

10. The process of claim 1 wherein the ultrafiltration concentrates the dilute solution containing IgG to a volume which is from about 1/10 to about 1/30 of the volume of said dilute solution before said concentration.

11. The process of claim 1 wherein the ultrafiltration concentrates the dilute solution containing IgG to a volume which is from about 1/15 to about 1/20 of the volume before said concentration.

12. The process of claim 1 wherein the concentrated solution resulting from ultrafiltration is subsequently freeze-dried to produce a solid composition of matter containing IgG.

13. A process for the manufacture of a concentrated solution containing Rh immune globulin (Rh IgG) which is suitable for intravenous use which comprises diluting high-titer Rh human plasma with an equal volume of water, subjecting said diluted plasma to chromatographic separation by applying said diluted plasma to a DEAE-Sephadex A-50 or a QAE-Sephadex A-50 column, eluting said column with an equilibrating buffer comprising 0.025 M phosphate buffer of pH 7.5

to provide an eluate which is a dilute solution containing Rh IgG, adding sodium chloride and glycine to said dilute solution to provide a dilute solution containing Rh IgG having a concentration of 0.15 M sodium chloride and 0.1 M glycine therein, and then subjecting said dilute solution to ultrafiltration to provide a concentrated solution containing Rh IgG.

14. The process of claim 13 wherein the diluted plasma is applied to the column at a rate of 500 ml per hour and the equilibrating buffer is applied to the column at a rate of 500 ml per hour.

15. The process of claim 13 wherein the ultrafiltration provides a concentrated solution containing Rh IgG which is from about 1/10 to about 1/30 of the volume of the dilute solution before concentration.

16. The process of claim 13 wherein the ultrafiltration provides a concentrated solution containing Rh IgG which is from about 1/15 to about 1/20 of the volume of the dilute solution before concentration.

17. A process for the manufacture of a solid composition of matter containing Rh IgG which is suitable for intravenous use which comprises subjecting the concentrated solution prepared as defined in claim 13 to freeze drying to provide a solid composition of matter containing Rh IgG.

18. A process for the manufacture of a solid composition of matter containing Rh IgG which is suitable for intravenous use which comprises subjecting the concentrated solution prepared as defined in claim 14 to freeze drying to provide a solid composition of matter containing Rh IgG.

19. A process for the manufacture of a solid composition of matter containing Rh IgG which is suitable for intravenous use which comprises subjecting the concentrated solution prepared as defined in claim 15 to freeze drying to provide a solid composition of matter containing Rh IgG.

20. A process for the manufacture of a solid composition of matter containing Rh IgG which is suitable for intravenous use which comprises subjecting the concentrated solution prepared as defined in claim 16 to freeze drying to provide a solid composition of matter containing Rh IgG.

21. A concentrated solution containing IgG when prepared by the process of claim 1.

22. A concentrated solution containing IgG when prepared by the process of claim 2, 3 or 4.

23. A concentrated solution containing IgG when prepared by the process of claim 5, 6 or 7.

24. A concentrated solution containing IgG when prepared by the process of claim 8, 9 or 10.

25. A concentrated solution containing IgG when prepared by the process of claim 11.

26. A solid composition of matter containing IgG when prepared by the process of claim 12.

27. A concentrated solution containing Rh IgG when prepared by the process of claim 13.

28. A concentrated solution containing Rh IgG when prepared by the process of claim 14, 15 or 16.

29. A solid composition of matter containing Rh IgG

when prepared by the process of claim 17.

30. A solid composition of matter containing Rh IgG when prepared by the process of claim 18.

31. A solid composition of matter containing Rh IgG when prepared by the process of claim 19.

32. A solid composition of matter containing Rh IgG when prepared by the process of claim 20.

This invention relates to an improved process for the manufacture of human plasma fractions containing immune globulin (IgG). The fractions may be obtained in the form of a concentrated aqueous solution or as a solid composition of matter containing IgG and these fractions are useful for intravenous injection.

The human plasma to be used in the process of this invention may be normal human plasma to provide standard or normal human immune serum globulin (HSIG or NSIG) or it may be plasma which has a high-titer of particular antibodies such as Rh factor antibodies, rabies antibodies, tetanus antibodies or zoster antibodies.

The process of this invention is particularly suitable for the preparation of a concentrated solution containing Rh immune globulin (Rh IgG) or a solid composition of matter containing Rh IgG, the solution and the solid composition being useful for intravenous injection.

The prevention of Rh isoimmunization by the passive administration of anti-D is known and originally, high-titer anti-D plasma was given intravenously to the Rh-negative expectant mother at risk. Two problems associated with the use of raw plasma are low Rh-antibody content and the risk of transmitting hepatitis. These problems were resolved by the introduction of an Rh IgG prepared by the Cohn cold ethanol fractionation of plasma with a high anti-D content. Unfortunately, IgG prepared by the Cohn method tends to form aggregates and thus precludes its use by intravenous administration. Aggregates of IgG fix and activate complement in the same way as do antigen-antibody complexes. They could, if injected intravenously, produce severe adverse reactions. An IgG which can be administered intravenously has a particular advantage in the case of Rh prevention. Intravenous

injection results in a much more rapid appearance of the Rh antibody in the circulation as well as a higher maximum level. In addition, intravenous injection causes much less discomfort than the intramuscular route.

We have now found, and herein lies our invention, that an IgG preparation which is suitable for intravenous use can be prepared by treating a dilute aqueous solution containing IgG with a mixture of sodium chloride and glycine and then concentrating the solution by means of ultrafiltration. The concentrated solution thus obtained may be used as such for the preparation of intravenous solutions containing IgG or it may be freeze-dried to provide a solid composition of matter which can also be used to prepare intravenous solutions containing IgG.

The invention as claimed herein is a process for the manufacture of an immune globulin (IgG) preparation which is suitable for intravenous use which comprises treating a dilute solution containing IgG with a mixture of sodium chloride and glycine and subjecting said dilute solution to concentration by means of ultrafiltration to provide a concentrated solution containing IgG.

The dilute solution containing IgG used as starting material may be a solution obtained as an eluate from a chromatographic separation procedure using a column containing an ion exchange resin, for example a *DEAE-Sephadex A-50 or a *QAE-Sephadex A-50 ion exchange resin. Such a dilute solution containing IgG, and particularly one containing Rh IgG, is useful as starting material in that the final product containing IgG, preferably Rh IgG, obtained therefrom is relatively pure and in high yield from the original plasma. The final product has a relatively low content of aggregates and relatively low anticomplementary activity and is suitable for

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intravenous use.

The process of this invention will be described further with particular reference to the manufacture of Rh immune globulin (Rh IgG) according to the following procedure:

Materials. Membrane filters and ultrafilter membranes were purchased from Millipore Ltd. (Toronto, Canada). DEAE-Sephadex A-50 was purchased from Pharmacia (Canada) Ltd. (Montreal). All other chemicals were of ACS reagent grade.

DEAE-Sephadex column preparation of Rh immune globulin. Rh immune globulin was prepared by the fractionation of high-titer Rh human source plasma on columns of DEAE-Sephadex by the following method:

DEAE-Sephadex A-50 (380 g) was treated sequentially with 1 M HCl, 1 M NaOH, and 0.25 M potassium phosphate buffer, pH 7.5, in a 70-liter Bel-art tabletop Buchner funnel. Between each treatment the DEAE-Sephadex was washed with large volumes of pyrogen-free distilled water and finally suspended in 0.025 M phosphate buffer, pH 7.5, for autoclaving (121°C, 15 psi, for 30 min.). After cooling the DEAE-Sephadex was packed into a 15 x 50-cm glass column and equilibrated with 0.025 M phosphate buffer, pH 7.5.

A 4-liter pool of Rh plasma was diluted with an equal volume of pyrogen-free water and was applied to the DEAE-Sephadex column at 500 ml/h. When all the plasma had been applied the column was eluted with equilibrating buffer (0.025 M phosphate, pH 7.5) at the same flow rate. The column effluent absorption at 280 nm was monitored by a Uvicord I LKB-Production AB (Bromma, Sweden) and the 280-nm absorbing peak was collected in one vessel, sodium chloride and glycine were added to this effluent (Rh IgG solution) to a concentration of 0.15 M and 0.1 M, respectively, in the effluent. The dilute Rh IgG solution (about 14 liters) was concentrated to

1000 ml by ultrafiltration with a Millipore Pelicon System using a PTGC 000 05 cassette membrane (NMWL of 10,000/5 ft^2) (Millipore Ontario Canada). Column fractionation and effluent concentration were carried out at 4°C in a clean room. All equipment in contact at any stage of the production process was sterilized by autoclaving, ethylene oxide, or heating to 270°C for 1 h. Pyrogen-free water prepared by glass distillation of reverse osmosis water was used for all washings and for the preparation of buffers.

The concentrated Rh IgG was centrifuged at 5000 rpm for 30 min. in a Sorvall RC-3 and sterilized via serial filtration through 0.8-, 0.45- and 0.22-µm pore size membrane in a 142-mm stainless-steel membrane holder.

The anti-D concentration of the filtered concentrate was measured by an Auto-Analyzer technique. The concentrate was then filled into 5-ml serum vials in either one or the other of two concentrations and then lyophilized. The volume filled into each vial was sufficient to allow removal of in excess of either 120 or 240 µg anti-D; the first concentration being for postpartum administration, the second for antenatal administration. All the equipment in contact with the product at any stage was sterilized by oven, steam, or ethylene oxide gas.

The purity, anticomplementary activity, safety (sterility and toxicity), hepatitis B surface antigen test, residual moisture, quantitative determination of IgG subclasses and anti-A, -B, -C and -E levels in the lyophilized product were determined according to established procedures before the product (*"WinRho") was used in clinical trials. Results:

*Preparation of Rh immune globulin, *"WinRho".* Plasma was diluted with an equal volume of pyrogen-free distilled water prior to fractionation to reduce its ionic strength to

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that of the eluting buffer (0.025 M phosphate, pH 7.5). The fractionation of the diluted plasma on the column of DEAE-Sephadex A-50 resulted in the adsorption of all the plasma proteins except Rh IgG which was eluted in a very dilute solution (about 0.2 g%). Ultrafiltration of the Rh IgG in the phosphate buffer alone resulted in poor solubility after lyophilization. However, the addition of sodium chloride and glycine to the column effluent significantly reduced the amount of precipitate formed during concentration. The sodium chloride and glycine stabilized the dilute Rh IgG during ultrafiltration and subsequent freeze-drying resulted in a readily soluble lyophilized Rh IgG. The Millipore cross flow ultrafiltration system appeared to produce minimum damage to the shear-sensitive IgG molecule in the presence of sodium chloride and glycine.

Recoveries of anti-D (as measured by an Autoanalyzer) in the final concentrated Rh IgG solution following filtration averaged 91%. This high yield was not, however, an indication of the recovery of all of the IgG from the column since only 65 to 75% of all the IgG in the starting plasma was eluted from the DEAE-Sephadex.

Safety. The rigid compliance to the asceptic protocol set out for the production of "WinRho" has resulted in all lots being sterile, nonpyrogenic, and nontoxic according to the test methods required by the Health Protection Branch, Department of Health and Welfare, Canada.

Although the usual human dose of "WinRho" would be the content of one 120- or 240-µg vial, certain clinical indications may require administration of several vials of "WinRho". The safety of "WinRho" was investigated by carrying out an LD_{50} (half the lethal dose) experiment. Vials of "WinRho" were reconstituted with 0.3 ml of 0.9% NaCl instead

of the recommended 2.0 ml and several mice were injected intravenously with varying doses of up to 1.25 ml per mouse. The usual safety test dose is 0.5 ml of "WinRho" dissolved in 2.0 ml with observation of the mice for 7 days. All mice had normal weight gains and only the 1.0- and 1.25-ml dose caused convulsions which lasted 2 and 5 min., respectively. The 1.25-ml dose was the equivalent of the injection of eight vials (1000 µg anti-D) of "WinRho" to a 20-g mouse. The LD_{50} (lethal dose) of "WinRho" was not determined since no mice died. This test indicated that very large doses are safe for human use.

"WinRho" has also been shown to be safe in regard to the transmission of hepatitis B. All Rh source plasma fractionated into "WinRho" was obtained by the plasmapheresis of naturally immunized Rh-negative female volunteers by the Winnipeg Rh Institute under the direct supervision of the Medical Director of the Institute. The medical history of each donor is known and in each case there has been no clinical evidence of hepatitis. Each bag of Rh plasma is negative for HB_3Ag when tested by the Abbott(Ausria II) RIA test as is each lot of final lyophilized Rh immune globulin.

Stability. The relative Rh antibody to total protein ratio for "WinRho" is about 1.2%. In order to fill the vials with volumes of 1.0 to 2.0 ml the protein concentration of the Rh immune globulin solution containing 120 and 240 µg would be about 1 and 2 g%. This protein concentration is too low to allow storage of "WinRho" as a liquid and retain stability of anti-D activity for as long as 1 year. Lyophilization immediately after production of the Rh IgG provided a solid composition of matter. With the maintenance of residual moisture levels below 3% the anti-D activity of "WinRho" remained stable and fragmentation and aggregation remained below 3% for at least 18 months at +4°C., and at least 18

months at room temperature.

Clinical trials. A detailed report of the preclinical and clinical trial of "WinRho" has been published. The combination of low protein, low anticomplementary activity and very low contamination with IgA make "WinRho" suitable for intravenous as well as intramuscular administration. Our studies showed that intravenous administration of "WinRho" resulted in the appearance of maximum levels of anti-D in the serum of volunteers within 1 h after administration compared with 2 to 3 days following the intramuscular injection of the same dose. Peak anti-D levels were twice as high as when the same dose was given intramuscularly.

The clinical trial consisted of an intravenous injection of "WinRho" at 28 weeks gestation to every unimmunized Rh-negative woman whose husband was Rh-positive or Rh unknown, followed by a second injection within 72 h after delivery of an Rh-positive baby. Our studies showed that a 240-µg anti-D dose resulted in a demonstrable level of Rh antibody for at least 12 weeks and that a 120-µg dose was sufficient for protection after delivery of an Rh-positive baby. The clinical trial showed "WinRho" to be a safe and effective means of prevention of Rh immunization.

The DEAE-Sephadex A-50 or QAE-Sephadex A-50 column method is very suitable for the production of the dilute aqueous solution of IgG used as starting material in the process of this invention. The procedure is relatively simple and the column technique lends itself to small scale production of hyperimmune globulin. The only pretreatment of the Rh plasma required before application to the column is reduction in ionic strength to that of the eluting buffer by dilution with pyrogen-free distilled water. Dilution of the plasma results in additional dilution of the Rh IgG eluted

from the DEAE-Sephadex or QAE-Sephadex column. We have found that the tendency for precipitation of Rh IgG during ultrafiltration concentration of the dilute solution is significantly reduced by stabilization of the dilute Rh IgG by addition of sodium chloride and glycine to the column effluent to a concentration of 0.15 M and 0.1 M respectively in the effluent. The use of sodium chloride and glycine has been found by us to be a very effective and improved process for the stabilization of the dilute IgG as well as for increasing the solubility of lyophilized IgG preparations.

It will be understood that the degree of concentration of the dilute IgG solution by ultrafiltration may vary according to the type of immune globulin product being prepared. In the case of Rh IgG, the concentration by ultrafiltration may be of the order of from about 1/10 to about 1/30, preferably from about 1/15 to about 1/20. In the case of anti-rabies or anti-Zoster immune globulin, the concentration may be of the order of from about 1/10 to about 1/100.

The use of an enclosed sterile column and sterile plastic tubing completely isolates the plasma and effluent from the outside contaminated environment. This together with the sterilization procedures and elimination of pyrogens significantly reduces the hazard of bacterial or pyrogen contamination of the final product.

Characterization of the product produced by the ion-exchange column shows that the Rh IgG is highly purified and is suitable for intravenous administration. Both immunoelectrophoresis and double-diffusion analysis indicate a pure IgG with no evidence of other plasma proteins. IgA, which is a frequent low-level contaminate of Cohn ethanol-produced IgG fractions, could not be found in "WinRho" when assayed by the low-level RID technique. Thus the concentration of IgA in

"WinRho" must be 0.01 g/liter or less which is less than 10% of the lowest concentration of IgA (0.1 g/liter) found in Cohn prepared IgGs produced by 13 different manufacturers.

Sedimentation velocity experiments demonstrated the homogeneity of the IgG produced by the ion-exchange method. A single symmetrical peak was observed with a sedimentation coefficient in the 6.0 S range. The purity and recovery of IgG from the DEAE-Sephadex or QAE-Sephadex column depends upon the ionic strength and pH of the eluting buffer. The use of 0.025 M phosphate buffer, pH 7.5, results in a high-purity IgG but a low recovery of IgG from the plasma (70-80%). However, the yield of Rh antibody in the preparation was consistently above 90%. The high yield of Rh activity relative to total IgG is not unexpected since IgG consists of a heterogeneous group of proteins classified into four subclasses according to their types of heavy chains. Studies of the IgG subclasses of anti-D indicate that it is restricted to the IgG_1 and IgG_3 subclasses. Our observation that IgG produced by our method contains a higher percentage of IgG_1 and IgG_3 and no IgG_4 compared with normal plasma is in agreement with these studies. The loss of IgG during column fractionation appears to be due to the loss of the components with lower isoelectric point subclasses IgG_2 and IgG_4, which contain only a small percentage of anti-D.

Both precipitation of IgG and exposure to ethanol have been implicated as factors in the development of aggregates and anticomplementary activity in Cohn prepared IgG. The present method of this invention separates IgG from other plasma proteins without the use of ethanol or any other precipitation procedure. We have found that Rh IgG produced by the ion-exchange method and use of our stabilization process using sodium chloride and glycine, has a very small content

of aggregates and very low-anticomplementary activity making it suitable for intravenous use. Our studies have shown that Rh IgG, ("WinRho"), when injected intravenously, not only appears in the circulation more rapidly but peak levels are twice as high as when it is given intramuscularly. Rh IgG, ("WinRho"), in solution is not stable in terms of anti-D content for as long as one year when stored at 4°C. but lyophilization results in a solid product which is stable for up to 18 months when stored at 4°C. and can be dissolved readily when required for intravenous use.

The above process involving the use of a column ion-exchange method using, for example, DEAE-Sephadex A-50 or QAE-Sephadex A-50 as the ion exchange resin, for the preparation of a dilute solution of IgG and subsequent treatment with a mixture of sodium chloride and glycine as a stabilizer followed by concentration by ultrafiltration provides a concentrated solution of purified IgG. The latter may be used to provide suitable preparations for intravenous use or it may be lyophilized to produce a solid composition of matter containing IgG for intravenous use. This process is clearly the method of choice for the preparation of Rh IgG (anti-D) in the form of the product "WinRho".

It will be understood by one skilled in the art that the process of the present invention may likewise be applied as the process of choice for other immune globulin (IgG) products for clinical use such as standard or normal human immune serum globulin (HSIG or NSIG), anti-tetanus immune serum globulin, anti-rabies immune serum globulin and anti-Zoster immune serum globulin.

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